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(71) Applicant *(for all designated States except US)*: DSM N.V. [NL/NL]; Het Overloon 1, NL-6411 TE Heerlen (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LANGEVELD, Pieter, Cornelis [NL/NL]; Venezuelastraat 55, NL-2622 BP Delft (NL). STARK, Jacobus [NL/NL]; Joost van Ospad 31, NL-3069 ZM Rotterdam (NL).

(74) Agents: MATULEWICZ, Emil, Rudolf, Antonius et al.; DSM N.V., DSM Patents & Trademarks, Office Delft (994-0760), P.O. Box 1, NL-2600 MA Delft (NL).

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(54) Title: DETECTION OF ANTIMICROBIAL RESIDUES IN EGGS

(57) Abstract: The present invention relates to a novel method for the rapid detection of the presence or absence of antimicrobial residues in eggs. A one step test method is described in which residues of antimicrobial compounds such as antibiotics are detected while inhibiting compounds naturally present in samples obtained from eggs, which may interfere with the test, are inactivated.

DETECTION OF ANTIMICROBIAL RESIDUES IN EGGS

Field of the invention

The present invention relates to a novel method for the rapid detection of the presence or absence of antimicrobial residues in eggs.

Background of the invention

The presence of antimicrobial residues in food and feed is a growing concern among the consumers due to health-related problems and the increase of drug resistant bacteria. Antibiotics are not only applied as medication, but certainly in case of poultry also widely used as antimicrobial growth promoting substances. It is well known that concentrations of antimicrobial residues in eggs may be high. In most countries, such as the countries of the European Union, Canada and the United States, Maximum Residue Levels (MRL) are regulated by legislation.

Test methods to detect antimicrobial residues in body liquids such as milk or urine such as microbial inhibition tests (e.g. agar diffusion tests) or methods making use of selective binders (e.g. antibodies or tracers) are well known. Examples of microbiological test methods have been described in GB-A-1467439, EP 0005891, DE 3613794, CA 2056581, EP 0285792 and US 5494805. These documents all deal with ready to use tests that make use of a test organism. The test organism is mostly imbedded in an agar medium, which may contain an indicator, a buffer solution, nutrients and substances to change the sensitivity for certain antimicrobial compounds in a positive or negative way.

Examples of suitable test organisms are strains of *Bacillus, Streptococcus* or *E.coli.* In general, the principle of these tests is that when antibacterial compounds are present in a sample at a concentration sufficient to inhibit the growth of the test organism the color of an acid/base or redox indicator will stay the same. However when no inhibition occurs, growth of the test organism is accompanied by the formation of acid or reduced metabolites leading to a change in the colour of the indicator.

These test methods are suitable for the detection of antimicrobial residues in body liquids. However up to now detection of antimicrobial residues in eggs was not possible due to the presence of antimicrobial substances, such as lysozyme, which are naturally present in high concentrations in eggs. These inhibiting substances

show inhibitory activity against the test micro-organism leading to false positive results.

In case of e.g. a milk or urine sample inhibiting substances such as lysozyme or lactoferrin can be inactivated by heating the sample, e.g. at 80°C for 10 minutes (Vermunt et. al., Netherlands Milk and Dairy Journal 47: (1) 31 – 40 (1993)), or by using well known dialysis methods (van Wall, Archiv für Lebensmittelhygiene 29: (6) 235 (1978)). After this pre-treatment the liquid sample can be used for further testing simply by following the procedures of the test. In case of a Delvotest® type of test (EP 0005891) the liquid sample can be added directly to the test, after which the test is incubated.

However heating of an egg sample at temperatures sufficient to inactivate inhibiting substances of the egg, such as lysozyme, always leads to coagulation of the sample. It was believed that such samples are not suitable for further processing anymore.

Up to now after heating at a temperature sufficient to inactivate antimicrobial substances other than antimicrobial residues, the antimicrobial residues to be detected have to be extracted from the coagulated egg sample. These extraction methods not only cost a lot of time and extra handling but even worse always lead to loss of at least part of the antimicrobial residues, if present in the sample (Inglis et. al., Journal for the Association of Official Analytical Chemists 61: (5) 1098 – 1102 (1978); Katz et. al., Journal of the Association of Official Analytical Chemists 61: (5) 1103 – 1106 (1978); Janetschke et. al., Monatshefe für Veterinaermedizin 34: (21) 824 – 826 (1979); Steiner, Monatshefe für Veterinarmedizin 45: (11) 382 – 386 (1990)). This may lead to false negative results and therefore antibiotics in consumer eggs, which of course is unacceptable from a health point of view. Moreover laboratories executing studies concerning the presence or absence of antimicrobial residues in foods are limited by the time available to execute these studies. With the present time consuming methods only a very limited amount of egg samples can be examined. Further, these assays can only be executed in well-equipped laboratories and by well-educated persons, which is also a limiting factor.

It can be concluded that up to now no suitable test method for the detection of antimicrobial residues in egg samples is available. The present methods are unreliable, time consuming and may lead to both false positive and false negative results, which in turn leads to unacceptable amounts of antibiotics in the food chain and to economic losses.

Detailed description of the invention

The present invention provides a reliable and simple to carry out, one-step test for the detection of antimicrobial residues in eggs.

Unexpectedly it has been found that when an egg sample is added to a test suitable for detecting antimicrobial residues and then is incubated for a sufficient time at a sufficient temperature to inactivate natural inhibiting compounds of the egg, the test can be incubated directly after heating to determine the presence or absence of antimicrobial residues.

It has been surprisingly found that antimicrobial residues diffuse directly from the coagulated egg sample into the test system. Thus additional extraction methods to obtain the antimicrobial residues from the coagulated egg sample are not required.

According to the invention there is thus provided a process for determining the presence of an antimicrobial residue in an egg which process comprises:

(i) contacting the sample with a test suitable for determining the presence or absence of an antimicrobial residue in the sample;

(ii) heating the contacted sample and test for a sufficient time interval to inactivate a natural inhibiting compound e.g. lysozyme present in the sample; and

(iii) incubating the contacted sample and test.

By natural inhibiting compounds are meant compounds which may disturb the test and which are naturally present in the sample such as naturally inhibiting compounds, for example lysozyme. Thus by disturbing or inhibiting is meant the behaviour of the compound on parts of the test, for example the test micro organism.

The invention also provides test kit for determining the presence or absence of an antimicrobial residue in a sample of an egg, which test kit comprises:

(i) a test suitable for determining the presence or absence of an antimicrobial residue in a sample; and

(ii) the sample,

wherein natural inhibiting compounds e.g. lysozyme present in the sample have been inactivated.

The exact time/temperature requirements depend on e.g. the condition of the sample (e.g. the starting temperature, the volume of the sample, whole egg, egg white or egg yolk); the type of test (e.g. microbial inhibition tests or assays based on selective binders (e.g. antibodies or tracers)); or the microorganism used in the test

(e.g. thermophilic or non-thermophilic *Bacillus* or *Streptomyces* species). Of course it should be taken care of that the heat treatment will not inactivate the antimicrobial residues to be detected. The heat treatment can be executed using any method known in the art, e.g. by heating in a water bath or by using an incubator as described below.

For example the test can be performed in the following way:

1. A sample of the egg is obtained by making a hole in the egg of e.g. approximately 1-2 square cm, prick the egg-yolk, place the egg with the hole down on a bottle, after the egg is empty the bottle is closed and the sample is homogenized by shaking. Alternatively of course any other method known in the art to obtain a sample of the total egg, egg white or egg yolk can be used;
2. Add a sufficient amount of the egg sample to be tested to a test using well known methods;
3. Heat the test, e.g. for approximately 10 minutes at 80° C, to inactivate the natural inhibiting substances (e.g. lysozyme), to coagulate the egg sample;
4. Incubate the test following the standard procedures of the test and read the result.

Any test suitable for determining the presence or absence of antimicrobial residues may be used in a process of test kits of the invention. Examples are described in GB-A-1467439, EP-0005891, DE-3613794, CA-2056581, EP-028579 and US 5,494,805 which are incorporated herein by reference. Suitable tests are those in which selected sensitive microorganisms are used, e.g. microbial agar diffusion tests, or tests based on selective binding of the compound to be detected. Selective binding can be achieved using the well-known antibody technology or by using specific tracers. An example of a specific tracer is the penicillin binding protein, which is used in e.g. the Delvo-X-Press® for detecting beta-lactams.

Examples of suitable microbial agar diffusion tests are tests in which species of *Bacillus, Streptococcus or E. coli* are used. Preferably thermophilic species, e.g. *Bacillus stearothermophilus* and *Streptococcus thermophilus* are used. Examples of preferred strains are *Bacillus stearothermophilus var. calidolactis C953* (deposited with the Laboratory of Microbiology of the Technical University of Delft under the accession number LMD 74.1 in 1974 and with the Centraal Bureau voor Schimmelcultures (CBS), Baarn under the accession number CBS 760.83 in 1983

were the strain is available to the public) and *Streptococcus thermophilus T101* (DSM 4022, deposited on March 3, 1987). Both strains are very sensitive to antimicrobial compounds, especially chemotherapeutics such as sulfa compounds and antibiotics such as penicillins and tetracyclines. *E.coli* strains or other suitable gram-negative bacteria can be used for the detection of e.g. quinolones.

Bacillus stearothermophilus var. calidolactis C953 and *Streptococcus thermophilus T101* are fast growing and have the advantage that they are thermophilic. For example the optimum growth temperature of said *Bacillus* strain is from 50° to 70°C. The test organism is therefore very suitable for a test according to the invention as it is not killed by heating to inactivate the natural inhibiting compounds which may be present in the egg sample.

When the test organism is a *Bacillus* strain, it is preferably incorporated into the agar medium in the form of a spore suspension which may be prepared and incorporated into the agar medium prior to solidification by known methods (see for example GB-A-1467439). When the test organism is a *Streptococcus* strain, the bacteria are preferably incorporated into the agar medium in the form of bacterial cells which may be prepared according to known methods (see for example EP 0285792). The concentration of the test organism in the agar medium is preferably between 10^5 and 10^{10} colony forming units per ml of agar medium.

Suitable nutrients to enable multiplication of the test organism in the absence of antimicrobial residues are for example assimilable carbon sources (e.g. lactose, glucose or dextrose), assimilable nitrogen sources (e.g. peptone) and sources of growth factors, vitamins and minerals (e.g. yeast extract).

The growth of the test microorganism can be detected using well known methods, preferably by colour change of the agar medium of the test sample. Typically a colour indicator, preferably an acid-base or a redox indicator, is used. Examples of suitable acid-base indicators include bromocresol purple and phenol red. Examples of suitable redox indicators include brilliant black, methylene blue, toludine blue and nile blue. Also combinations of two or more indicators can be used.

Optionally the sensitivity of the test may be altered by adding certain substances, by changing the test conditions such as pH or concentration of buffering substances or agar or by varying the ratio of the volumes of agar and egg sample. Examples of substances that may be added to the test system to change sensitivity are nucleosides such as adenosine, or antifolates such as trimethoprim, ormethoprim or tetroxoprim, which improve the sensitivity of the test organism to

sulfa compounds. Salts of oxalic acid or hydrofluoric acid may be added to improve the sensitivity to tetracyclines. Cysteine may be added to diminish the sensitivity to penicillins.

The amount of egg sample (whole egg, egg white or egg yolk of any species, preferably poultry) to be added to the test depends on the test system. For microbial diffusion tests typically from 0.01 to 1.0 ml, preferably from 0.05 to 0.5 ml is added to the test using well-known methods. After addition of the egg sample the test is heated to inactivate the natural antimicrobial compounds present in the sample, e.g. lysozyme, of the egg sample. Preferably the test is heated for from 2 to 20 minutes at from 70°C to 100°C, more preferably the test is heated for from 10 to 15 minutes at from 75°C to 85°C or for from 2 to 6 minutes at about 100°C. Any other time / temperature treatment, which is sufficient to inactivate the natural inhibiting compounds of the egg without inactivating the antimicrobial residues to be detected, may be used.

After the heat treatment the test is incubated following the instructions of the test manufacturor. The incubation time of the test is dependent on the circumstances. In case of an agar diffusion tests using *Bacillus stearothermophilus* the test is incubated in a water bath or block heater at from 60°C to 70°C, preferably at from 62°C to 65°C. Typically, results may be obtained after 1.5 to 4 hours, preferably from 2.5 to 3.5 hours. In case of tests using selective binders, such as antibodies or tracers, the results may be obtained within about 30 minutes.

Conventional microbial inhibition tests suitable for use in the present invention include the commercial products, Delvotest®, Premi®test and BR-test® (obtainable from DSM N.V. Holland) the ADM Copan®test (Copan, Italy) and the Charm®AIM test (Charm, USA). Inactivation of the natural inhibiting compounds present in the egg sample, e.g. lysozyme, is preferably achieved by heating for example for from 5 to 15 minutes at for example from 75° C to 85°C. Alternatively any other temperature / time treatment, which is sufficient to obtain said effects, can be used.

In a further aspect, the invention provides a test kit for carrying out the method of the invention. This test kit contain the test and is suitable to execute the method of the invention: add the egg sample, heat to inactivate the natural inhibiting compounds of the sample, incubate the test and read the results.

Examples of kits useful for the purpose of the invention are transparent tubes, single or in a set, or combined to a block of translucent material provided with

a number of holes shaped therein (incubator). The test kit may contain solidified agar medium which may be optionally buffered; a test organism (e.g. a strain of *Bacillus* or *Streptococcus*) at sufficient colony forming units; nutrients for growth of said organism; an indicator (e.g. an acid-base or redox indicator); optionally substances to change the sensitivity for certain antimicrobial compounds in a positive or negative way. All ingredients may optionally be added to the test as a separate source, for example as a tablet or paper disc.

The test kits preferably have determined sizes. This is because of the reliability of the test. In case of a test based on agar diffusion technology, preferably tubes are used. The test unit will preferably be high enough to contain an amount of agar medium and a sample corresponding to preferably a height of from 3 to 30 mm, more preferably from 5 to 15 mm. The internal cross-sectional dimension of the test units is preferably from 1 to 30 mm, more preferably from 5 to 15 mm. The test units are preferably closed air tight during storage in which conditions they may be stored for at least several months. Of course any other test unit suitable for executing the method of the invention is included in this invention.

The volume of the agar medium in the test unit is determined by the height of the test unit, the internal cross-sectional dimension of the test unit and the percentage of the volume of the test unit, which is filled with the agar medium. The volume of the agar medium is preferably from 10 µl to 5 ml, more preferably from 100 µl to 1 ml.

Incubators suitable to execute the heat treatments as described in this invention can be constructed in such a way that after placing the test units in the incubator, heat and incubation treatments as described above can be done. The first heat treatment to inactivate the inhibiting compounds and optionally to form solid matrix and / or to activate the spores is executed at a higher temperature, after which the incubation of the test may continue at a lower temperature. Optionally after the incubation of the test the incubator can cool down to a temperature sufficient to stop the test.

An example of such an incubator is a block heater in which test units (e.g. ampoules) can be placed. For example in case of a conventional microbial agar diffusion test using a *Bacillus stearothermophilus* strain the incubator / block heater may contain a number of holes suitable for placing the test ampoules or test plates (e.g. Delvotest® or Premi®Test) therein. After placing the ampoules or plates the incubator heats the test to a temperature of e.g. from 75°C to 85°C for e.g. from 10

to 20 minutes after which the incubator turns to a lower temperature of from 62°C to 65°C for from 1.5 to 4 hours (incubation of the test). Of course the exact time / temperature intervals depend on many factors and will differ per type of test. This invention includes all incubators capable to execute a pre-incubation at a certain temperature for a certain period of time directly followed by an incubation at a lower temperature for a certain period of time. Optionally after the incubation of the test the incubator can cool down to a temperature sufficient to stop the test.

The process described in this invention is very simple to carry out, so that persons who perform the test do not have to be specially educated or trained.

All documents mentioned in this application are herein incorporated by reference to the same extent as if each individual application or patent was specifically and individually indicated to be incorporated by reference.

Example 1

Preparation of a whole egg sample

To obtain an egg sample for examination on the presence or absence of antimicrobial residues a hole of approximately 1-2 cm^2 was made in the egg, the egg yolk was pricked and the egg was placed with the hole down on a bottle allowing the egg white and egg yolk to drip into the bottle. After the egg was emptied, the bottle was closed and the sample was homogenized by shaking.

Example 2

Inactivation of natural inhibiting compounds of the egg and examining the samples on Delvotest®

Samples of 5 eggs (duplicate), which did not contain antimicrobial residues, were obtained according to the method described in Example I. To inactivate the natural inhibiting compounds present in the egg sample, 100 µl of each of the 5 samples was added on Delvotest® ampoules. The test was produced according to the methods described in EP 0005891 with the nutrients present in the agar. After heating for 10 minutes at 80°C in a waterbath, the ampoules were immediately placed in a waterbath at 64°C and incubated following the instructions of the producer. After 140 minutes the colour of all tests turned from purple to yellow, indicating that no antimicrobial rediues were present.

Control samples were not heated at 80°C for 10 minutes, but directly placed on the ampoule. These tests remained purple for at least 4 hours.

These results clearly demonstrate that natural inhibiting compounds in the egg sample inhibited the test leading to false-positive results. When the sample was heated as described above, the activity of the natural inhibiting compounds was eliminated and no false-positive results were observed anymore.

Example 3

Determination of the sensitivity of the Delvotest® according to the method described in this invention using spiked samples

Egg samples were obtained according to the method described in Example I. The samples were spiked by adding Penicillin G (0 and 4 ppb) or Sulphadiazine (0 and 100 ppb). The egg samples were added to Delvotest® ampoules (see Example 2) according to the method described in this invention: heated for 10 minutes at 80°C, and then immediately placed in a waterbath at 64°C and incubated following the instructions of the manufacturer. The results were read as soon as the colour turned to yellow (after 140 minutes). The samples containing no Penicillin G (o ppb) or Sulphadiasine (0 ppb) were negative, while the samples spiked with 4 ppb Penicillin G and 100 ppb sulphadiazine remained purple (positive).

These results clearly demonstrate that the method descibed in this invention is suitable for detecting antimicrobial residues in egg samples.

CLAIMS

1. A process for determining the presence or absence of an antimicrobial residue in a sample of an egg which process comprises:
(i) contacting the sample with a test suitable for determining the presence or absence of an antimicrobial residue in the sample;
(ii) heating the contacted sample and test for a sufficient time interval to inactivate a natural inhibiting compound, for example lysozyme, present in the sample; and
(iii) incubating the contacted sample and test.

2. A process according to claim 1, wherein the contacted sample and test are heated to a temperature of from 70°C to 100°C.

3. A process according to claim 2, wherein the contacted sample and test are heated to a temperature of from 75°C to 85°C.

4. A process according to any one of claims 1 to 3, wherein the sample and test are heated for from 2 to 20 minutes.

5. A process according to claim 4, wherein the sample and test are heated from 10 to 15 minutes.

6. A process according to claim 1 wherein the test comprises a test organism, nutrients and one or more indicators present in an agar medium.

7. A process according to claim 6 whereby the degree of growth or inhibition of growth of the test organism is determined indicating the absence or presence of the antimicrobial residue.

8. A test kit for determining the presence or absence of an antimicrobial residue in a sample of an egg, which test kit comprises:
(i) a test suitable for determining the presence or absence of an antimicrobial residue in a sample; and
(ii) the sample,

wherein lysozyme present in the sample has been inactivated.

9. Use of an antimicrobial inhibition test to test an egg sample on the presence of an antimicrobial residue.

10. Use of an incubator for an antimicrobial residue test to inactivate lysozyme in an egg sample.

11. A computor program in combination with a computor, whereby the program causes the computor to operate in such a way that the computor controls the temperaure of an incubator, whereby after starting the program, the temperature will be set at a temperature for a selected inactivation time interval whereby lysozyme will be inactivated in an egg sample, whereafter the temperature is set at a temperature whereby incubation of the test sample takes place during a selected incubation time interval.